Supporting Information

Zhang et al. 10.1073/pnas.1214156110

SI Materials and Methods

Cell Culture and Reagents. HEK293T, SaoS2, and HeLa cells were cultured in DMEM (GIBCO) supplemented with 10% (vol/vol) FBS. HCT116 WT, HCT116 $p53^{-/-}$, and U2OS cells were cultured in McCoy's 5A supplemented with 10% FBS. The following reagents were used: MG132 (Calbiochem), nocodazole (Sigma), imidazole (Sigma), GSH-Sepharose (GE Healthcare), Ni-Sepharose (GE Healthcare), doxorubicinorubicin (Sigma), and etoposide (Sigma).

Plasmids and Antibodies. Tripartite motif 39 (TRIM39), p21, and Cdt2 were cloned into expression vectors, and deletion mutants were generated by a PCR-based approach. All constructs were sequenced before use. For Western blot analysis, the following antibodies were used: anti-p21 (BD Pharmingen/Santa Cruz Biotechnology), anti-Cul4 (Santa Cruz Biotechnology), anti-Myc (Cell signaling technology; Santa Cruz Biotechnology), anti–DNA damage-binding protein 1 (DDB1; Bethyl), anti-Cdt2 (Bethyl), anti-Skp2 (Santa Cruz Biotechnology), anti-FLAG (Sigma), anti-HA (Roche), anti-phosphohistone H3 (Millipore), anti-puma (Sigma), anti-p27 (BD Pharmingen), anti-p53 (Santa Cruz Biotechnology), anti-Mdm2 (BD Pharmingen), anti–β-actin (Sigma), anti-proliferating cell nuclear antigen (Abcam), anti-His (Sigma), and anti-GFP (Sigma).

Real-Time PCR. Total RNA was extracted with TRIzol (Invitrogen). RNA (4 μg) was reverse-transcribed in a 20-μL reaction using the PrimeScript RT-PCR Kit (TAKARA). After RNase H treatment at 37 °C for 20 min and after inactivation by incubating samples at 95 °C for 2 min, the RT reaction was diluted. cDNA was used for RT-PCR or real-time PCR assay. Primer sequences were as follows:

TRIM39α: 5′-GGCTTCGAGATGCTTAA GGATGT-3′, 5′-CCCGTGGACAGATCGTTGA-3′

TaqMan probe: 5′FAM-CTCTCCTTGGATTAGTAAAA-3′ MGB; *TRIM39β*: 5′-AGGGTCACATCCGCAATTAG-3′, 5′-GTC AAAAGTACCCTGGAAAAATGTG-3′

TaqMan probe: 5′FAM-CCCTGG AAAAATGTGAAAA-3′ MGB

In Vivo Ubiquitylation Assay. HCT116 WT or HCT116 $p53^{-/--}$ cells expressing HA-ubiquitin were infected with indicated lentiviruses. Cells were treated with 20 μM MG132 for 6 h before being harvested by modified radioimmunoprecipitation assay buffer (50 mM Tris·HCl at pH 8.0, 0.1% SDS, 1% sodium deoxycholine acid, 1% Triton X-100, 0.15 M NaCl, 5 mM N-ethylmaleimide, 1 mM Na_4VO_3, 10 mM NaF, and 1 mM EDTA). Then ubiquitylation assay was performed as previously described (1).

Flow Cytometry. Cells were harvested and fixed in 70% ethanol. For cell cycle and apoptosis assay, the fixed cells were washed once with PBS and then incubated with PBS containing 10 μg/mL propidium iodide and 20 μg/mL RNaseA for 30 min at 37 °C. Cells were analyzed by flow cytometry using an Epics XL flow cytometer (Beckman Coulter).

Immunofluorescence. HCT116 $p53^{+/+}$ cells grown in 10-cm^2 dishes were transfected with TRIM39-Myc (2 μg). Twenty-four hours later, cells were split into new 10-cm^2 dishes with coverslips. At an additional 24 h later, the culture medium was removed and coverslips were carefully washed three times with PBS. Cells were then fixed with 4% paraformaldehyde for 5 min at room temperature and subsequently washed twice with PBS and twice with washing buffer. Cells were then permeabilized for 5 min with 0.5% Triton X-100, blocked in PBS plus 1% BSA, and subsequently incubated with appropriate primary/secondary antibodies and DAPI. Confocal fluorescence images were obtained with a confocal microscope (LSM 780 NLO; Carl Zeiss).

Immunohistochemistry. Formalin-fixed, paraffin-embedded 5-μm tissue sections were deparaffinized in xylenes and rehydrated through a graded series of alcohols. After antigen retrieval was performed, all sections were blocked at room temperature in avidin/biotin blocking buffer (Vector Laboratories) and then in 3% BSA for 30 min. Staining with anti-p21 antibody was conducted at room temperature for 60 min. Sections were rinsed twice in PBS, and protein staining was performed using a diaminobenzidine substrate kit. Samples were counterstained with hematoxylin. Immunohistochemistry images were obtained using an upright microscope (Nikon).

In Vitro Binding Assay. Bacterially expressed GST or GST-Cdt2 was bound to glutathione-Sepharose beads and incubated with purified His-p21 and/or His-TRIM39α, His-TRIM39β, or His–TRIM39-N in TN buffer (50 mM Tris·HCl at pH 8.0, 250 mM NaCl, 10 mM β-glycerol phosphate, 10 mM NaF, 1 mM Na_4VO_3, and 0.5% Triton X-100) for 1 h at 4 °C. The beads were washed with TN buffer and subjected to SDS/PAGE.

1. Kim Y, Starostina NG, Kipreos ET (2008) The CRL4Cdt2 ubiquitin ligase targets the degradation of p21Cip1 to control replication licensing. *Genes Dev* 22(18):2507–2519.

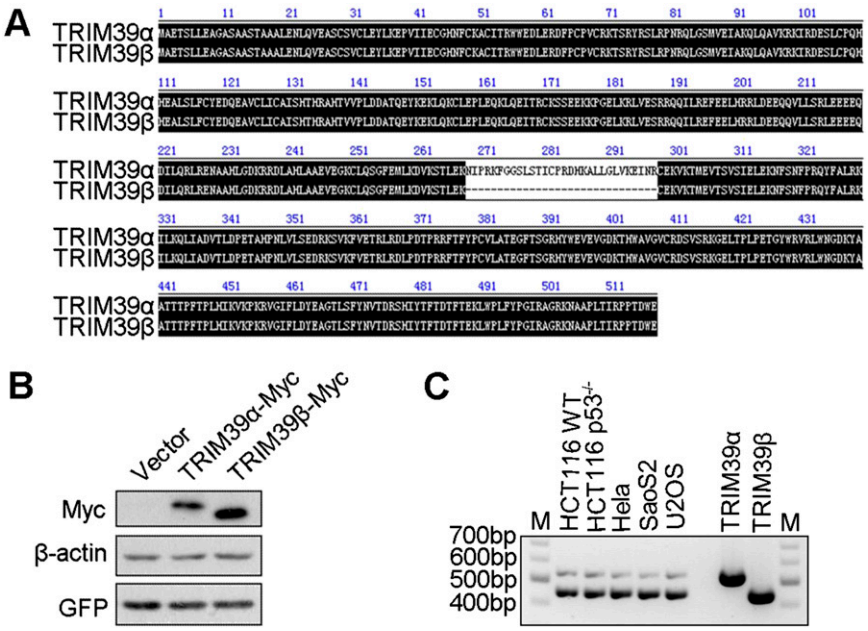

Fig. S1. Amino acid sequences, exogenously expressed protein abundance, and mRNA levels of TRIM39α and TRIM39β. (*A*) Comparison of the deduced amino acid sequences of TRIM39α and TRIM39β. Homologous amino acid residues are highlighted in the shaded sections. (*B*) HEK293T cells were transfected with indicated plasmids for 36 h. Cell lysates were collected and subjected to Western blotting with the indicated antibodies. (*C*) Total RNA extracted from HCT116 WT, HCT116 $p53^{-/-}$, HeLa, SaoS2, and U2OS cells was subjected to semiquantitative PCR analysis to determine the mRNA abundance of TRIM39α and TRIM39β. M, marker.

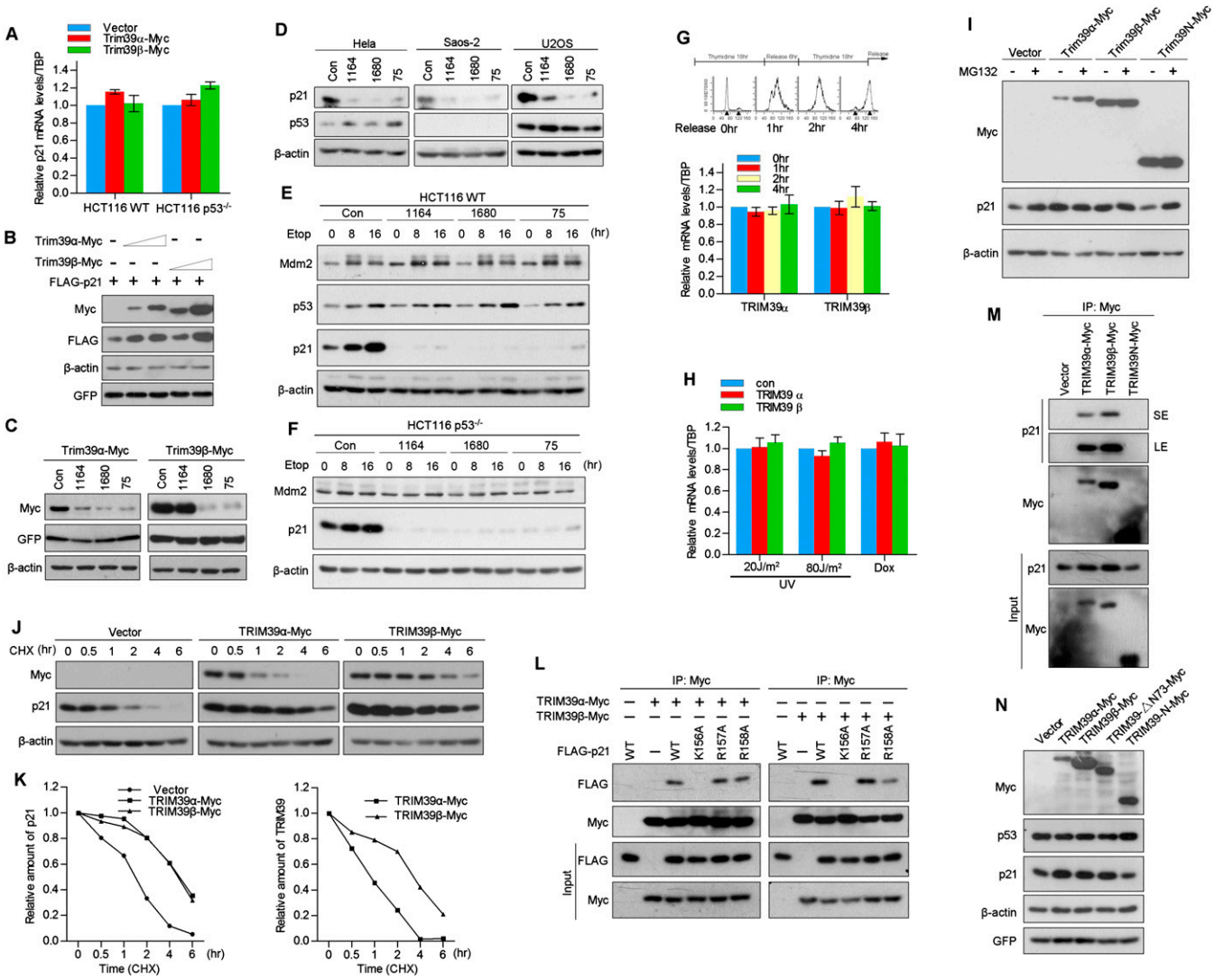

Fig. S2. TRIM39 stabilizes p21. (*A*) HCT116 WT and HCT116 $p53^{-/-}$ cells were infected with lentivirus encoding TRIM39α and TRIM39β. Total RNA was extracted and subjected to quantitative RT-PCR (qRT-PCR) analysis. TPB, TATA box binding protein. (*B*) HEK293T cells were transfected with FLAG-p21, along with TRIM39α-Myc or TRIM39β-Myc. Cell lysates were collected and subjected to Western blotting with the indicated antibodies. (*C*) HEK293T cells expressing TRIM39α-Myc or TRIM39β-Myc were transfected with the indicated TRIM39 shRNAs. Protein lysates were analyzed by Western blotting with the indicated antibodies. Con, control. (*D*) HeLa, Saos-2, and U2OS cells were infected with lentivirus encoding the indicated shRNAs. Proteins were then extracted and subjected to Western blot analysis. (*E* and *F*) HCT116 WT and HCT116 $p53^{-/-}$ cells were infected with lentivirus encoding the indicated shRNAs. Cells were then treated with 5 μM etoposide (Etop) for the indicated times, and proteins extracted were collected and subjected to Western blotting. (*G*) HCT116 WT cells stably expressing the indicated shRNAs were released from a double thymidine block. Total RNA was isolated and subjected to qRT-PCR analysis. Error bars represent the SD of triplicate measurements. (*H*) HCT116 WT cells infected with lentivirus encoding the indicated shRNAs were treated with UV irradiation (20 J/m^2 or 80 J/m^2) for 6 h or with 0.2 μM doxorubicin for 8 h. Total RNA was then isolated and subjected to qRT-PCR analysis. Error bars represent the SD of triplicate measurements. (*I*) HCT116 $p53^{-/-}$ cells infected with the indicated TRIM39 lentiviral constructs were treated with DMSO or MG132 for 4 h. Proteins were then extracted and subjected to Western blotting. (*J*) HCT116 $p53^{-/-}$ cells infected with the indicated lentivirus were treated with 25 μg/mL cycloheximide (CHX) for the indicated times. Lysates were harvested and analyzed by Western blotting. (*K*) Semiquantification was performed with β-actin as a loading control and relative p21 levels (*Left*) and relative TRIM39α or TRIM39β levels (*Right*) at time 0 set as 1. (*L*) HEK293T cells were transiently transfected with the indicated constructs. Total cell lysates were immunoprecipitated with the indicated antibodies. IP, immunoprecipitate. (*M*) HCT116 WT cells infected with the indicated lentiviral constructs were collected, and the cell lysates were immunoprecipitated with anti-Myc antibody. The immunoprecipitates were subjected to Western blotting with the indicated antibodies. (*N*) U2OS cells expressing the indicated TRIM39 constructs were collected, and protein extracts were analyzed by Western blotting with the indicated antibodies.

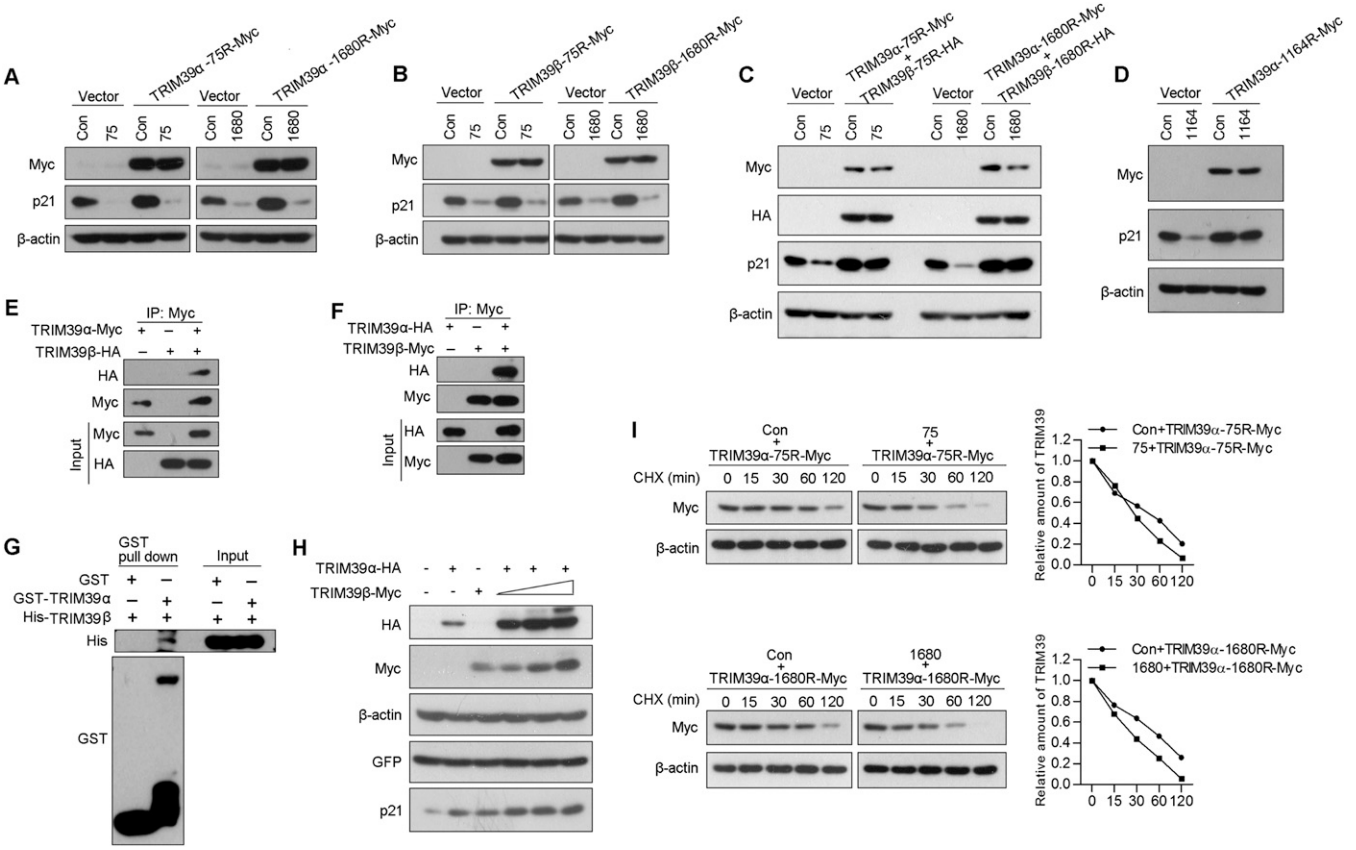

Fig. S3. TRIM39α and TRIM39β are both required to regulate p21 stability. (*A–D*) HCT116 $p53^{-/-}$ cells stably expressing shRNA-resistant forms of TRIM39α or/and TRIM39β were infected with indicated lentiviral shRNA constructs. Cells were then harvested, and lysates were subjected to immunoblotting with the indicated antibodies. Con, control. (*E* and *F*) TRIM39α interacts with TRIM39β in vivo. HEK293T cells transfected with the indicated constructs were lysed, followed by immunoprecipitation with anti-Myc antibody. The immunoprecipitates (IP) were then blotted with the indicated antibodies. (*G*) TRIM39α interacts with TRIM39β in vitro. Purified His-TRIM39β was incubated with GST or GST-TRIM39α coupled to GSH-Sepharose. Proteins retained on Sepharose were then blotted with the indicated antibodies. (*H*) TRIM39β stabilizes TRIM39α. HCT116 $p53^{-/-}$ cells infected with the indicated lentivirus were lysed, and lysates were blotted with the indicated antibodies. (*I*) HCT116 $p53^{-/-}$ cells infected with the indicated lentivirus were treated with 25 μg/mL cycloheximide (CHX) for the indicated times. Lysates were harvested and analyzed by Western blotting. (*Right*) Semiquantification was performed with β-actin as a loading control and relative p21 levels at time 0 set as 1.

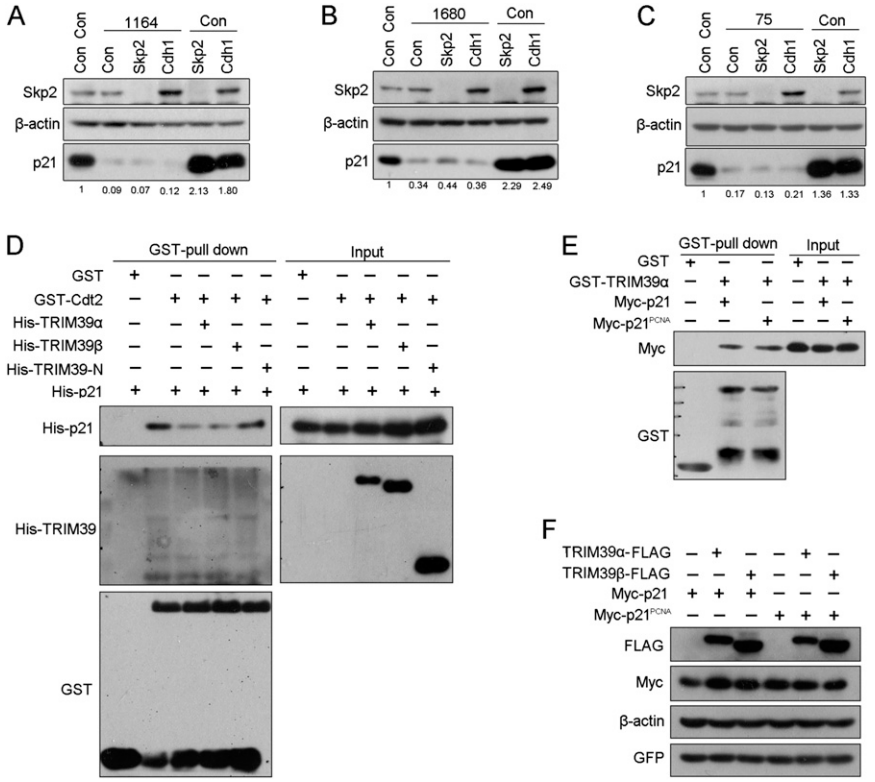

Fig. S4. TRIM39 protects p21 from ubiquitylation and degradation mediated by $CRL4^{Cdt2}$. (*A–C*) Depletion of Skp2 or Cdh1 failed to block p21 degradation in TRIM39-deficient cells. HCT116 WT cells were infected with lentivirus encoding the indicated shRNAs. Cells were then collected, and lysates were subjected to immunoblotting with the indicated antibodies. Con, control. (*D*) Absence of in vitro interaction between TRIM39 and Cdt2. Bacterially expressed GST or GST-Cdt2 was bound to glutathione-Sepharose beads and incubated with purified His-p21 in the presence or absence of His-TRIM39α, His-TRIM39β, or His–TRIM39-N in TN buffer. Proteins retained on Sepharose were blotted with the indicated antibodies. (*E*) Purified Myc-p21 or Myc-$p21^{PCNA}$ (proliferating cell nuclear antigen binding-deficient mutant of p21) was incubated with GST or GST-TRIM39α coupled to GSH-Sepharose. Proteins retained on Sepharose were then blotted with the indicated antibodies. (*F*) HEK293T cells were transfected with the indicated plasmids for 36 h. Cell lysates were collected and subjected to Western blotting with the indicated antibodies.

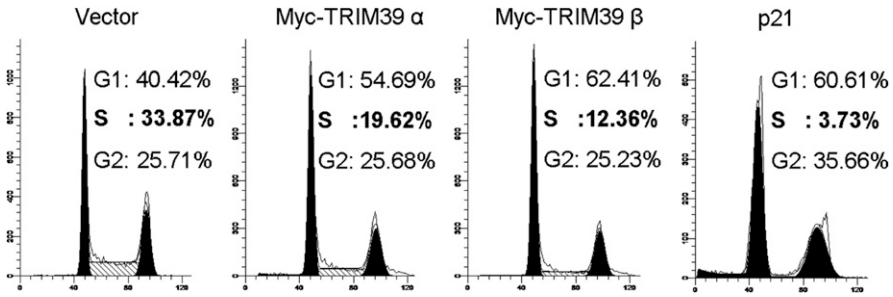

Fig. S5. TRIM39 regulates the G1/S transition. HCT116 WT cells infected with the indicated lentiviral constructs were collected, and the DNA content was analyzed by propidium iodide staining. Results are one trial representative of three independent experiments.